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PRINCIPAL INVESTIGATOR: Kent W. Hunter, Ph.D.

CONTRACTING ORGANIZATION: Fox Chase Cancer Center
Philadelphia, Pennsylvania 19111

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13. ABSTRACT *(Maximum 200 words)*

Metastasis is a complex phenomenon involving both the activation and inactivation of a number of genes. A great deal is known about the types and numbers of genes that are activated during the metastatic pathway, including proto-oncogenes, proteases, and growth factors. Relatively little is known, however, about the inactivation of genes that suppress metastatic potential. Identification and characterization of these genes would help provide additional insights into the biology of metastatic progression, as well as potentially leading to novel therapeutic strategies.

To identify metastatic suppressor genes, we have performed a genetic strain survey to identify inbred mouse strains that specifically inhibit the ability of a transgene induced mammary tumor to form pulmonary metastases. We have identified thirteen inbred mouse strains that suppress the ability of this tumor to disseminate, indicative of the presence of dominant suppressor genes. A range of suppression, between 2 and 30-fold was observed depending on the inbred strain. The wide variance observed is suggestive of a multigenic or complex trait type of inheritance. We have, therefore, initiated a number of backcross mapping experiments with the most interesting strains to begin to map the genomic location of the metastasis suppressor genes.

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Introduction

The existence of metastatic suppressor genes was originally predicted based on somatic cell hybrid fusions between nonmetastatic and metastatic tumor cells. The resulting hybrids, while retaining their tumorigenic potential, were unable to metastasize [1-3]. A prostate cancer metastatic suppressor locus was localized on human chromosome 11 in the region 11p11.2 and was subsequently shown to be KAI1, a leukocyte surface glycoprotein [4]. Analysis of murine melanoma and human breast cancer cell lines revealed the specific down regulation of the gene NM23, a nucleoside 5′-phosphate kinase in metastatic tumors or cell lines versus nonmetastatic samples [5]. Introduction of E-cadherin cDNAs into tumor cell lines has demonstrated the suppression of metastatic capacity of both mouse and human carcinomas [6-8].

Additional evidence for the existence of genes that can suppress metastasis was generated from a series of transfection experiments into murine cells. It was determined that a variety of activated proto-oncogenes, including H-RAS, v-mos, v-raf, A-RAF, v-src, v-fes, v-fms, and p53 could induce primary tumors with metastatic dissemination when transfected NIH-3T3 cells were injected into mice. However, when the same oncogenes were transfected into cell lines derived from different strains of mice, metastatic potential, but not tumorigenicity, was lost [9, 10]. This suggests that certain alleles present in some of the inbred strains of mice, either alone or in combination, can function as a metastasis suppressor. At present, these loci have yet to be characterized.

Although the mouse has been used a model for a number of individual steps in the metastatic cascade, a mouse model for the entire process has not been developed. This is because naturally occurring mouse tumors in general do not metastasize, possibly because the animal succumbs to the primary tumor before the metastatic process can be completed. A number of transgenic animals, however, have been found to metastasize, possibly due to the accelerated nature of the disease [11-22]. Since these animals develop metastatic disease in a heritable and highly penetrant manner, they offer the potential to utilize the power of mouse genetics to identify and characterize the modifier/suppressor loci known to be present in the mouse genome. One particularly interesting transgenic mouse is the mouse mammary tumor virus-polyoma middle T (MMTV-PyMT) transgenic animal, which develops mammary tumors and extensive pulmonary metastases [23]. The MMTV-PyMT transgenic mouse develops synchronously appearing multifocal tumors involving all of the mammary glands. Females develop palpable tumors within 8 weeks of birth, independent of pregnancy. Males also develop mammary tumors, although with a longer latency. In addition, more than 90% of the MMTV-PyMT animals develop hundreds of pulmonary metastases by 3 months of age. The high penetrance and extensive metastatic potential of this animal make it an excellent model to perform genetic screens for metastasis modifier/suppressor genes.

The purpose of this proposal is to utilize the MMTV-PyMT transgenic mouse as a model of human breast cancer metastasis in order to genetically localize, clone, and characterize genes that modulate or suppress the metastatic process. The differential ability of cell lines originating from different mouse strains to metastasize following the transfection of oncogenes suggests that

there is at least one major metastatic suppressor gene present in some strains, although additional genes may be detected with a broad strain survey.

The specific aims of this project are as follows:

1) Utilize the highly metastatic MMTV-PyMT transgenic animal to perform a mouse strain survey to determine the mouse strains that contain dominant metastatic modifier/ suppressor alleles by breeding the transgenic animal to a variety of mouse strains and scoring for latency, progression, extent and organ tropism of the mammary tumor metastases.
2) Select those mouse strains that demonstrate the largest effect on metastatic process and commence appropriate backcrosses to localize the gene or genes of interest as a preliminary step for isolation and characterization of the genes of interest, either by candidate gene or positional cloning strategies.
3) Initiate positional cloning projects to clone, identify and characterize the most promising candidate loci.

Characterization of Transgenic Metastasis Phenotype:

The (FVB/N-TgN(MMTVPyMT) transgenic animal was imported to the Fox Chase Cancer Center, and a colony established. The metastatic phenotype of our colony was assessed, was indistinguishable from the published phenotype, indicating that either the metastatic phenotype was independent of environmental factors, or that the same environmental factors existed in our animal facilities and the original facility. To develop a robust protocol for detecting metastatic suppresser genes in a mouse genetic screen, the affect of tumor duration, the period of time between diagnosis of the primary tumor and sacrifice, was determined. FVB/N-TgN(MMTVPyMT) virgin female animals were monitored until diagnosis of a primary mammary tumor was determined by daily visual inspection and palpation. The animals were subsequently aged to permit the potential development of metastases. The animals were then sacrificed and lungs were harvested from each of the animals and paraffin embedded for histological examination. Three nonadjacent sections from each lung, each separated by 100 microns, were hemotoxylin-eosin stained, and the pulmonary metastatic density was determined utilizing a Leica M420 low power microscope and the Q500MC Image Analysis System. The Leica Q500 Image Processing and Analysis System was utilized to exclude bronchial and alveolar space from the calculation, to minimize density variation that might arise from variable degrees of lung inflation at time of harvesting. Three randomly chosen fields were scored for each slide, for a total of 9 fields for each animal. Greater than 50% of the tissue area of each slide is estimated to have been scored. The number of metastases per unit area of lung was then plotted versus duration of tumor exposure. No significant trend was observed for increased tumor exposure. Since no significant difference was observed in the pulmonary metastatic density due to time, a time window of 30-40 days post-diagnosis was selected as the endpoint for the determination of pulmonary metastatic density.

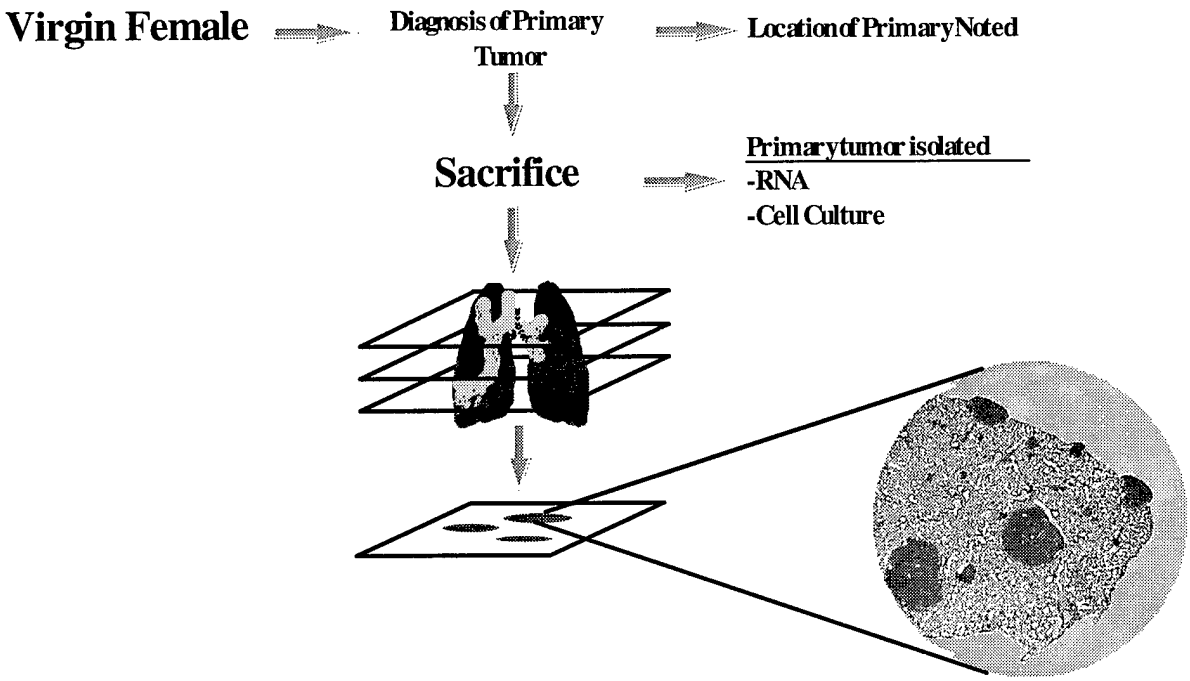

Figure 1: Protocol for Analysis of Pulmonary Metastasis. Virgin female transgenic animals are aged until induction of primary tumor, monitored to confirm presence of the tumor, and aged 30-40 days to permit metastatic progression. Primary tumor is harvested for Northern blot analysis and establishment of cell cultures. Lungs are harvested, sectioned, stained, and analyzed microscopically for number, size and density of pulmonary metastases.

To assess the effect of tumor burden on the metastatic phenotype, the correlation between pulmonary metastatic density and approximate tumor burden was determined. At diagnosis of the primary tumor, each animal was weighed, and then aged for 20-70 days. Animals were then sacrificed and total carcass weight was determined. A crude approximate of tumor burden was determined by subtracting weight at sacrifice by weight at diagnosis. Due to limited manpower and the large number of animals to be analyzed, it was not possible to dissect out the mammary tumors from all of the animals. Cachexia was not observed in any of the MMTVPyMT animals, therefore, the change of weight should be a result predominantly, although not exclusively, of tumor tissue accumulation. In addition, most mouse strains accumulate body weight at approximately the same rate [24], so the percentage of the total change of weight that is tumor should be approximately the same in each strain. Complete dissection of the tumors from a subset of these animals demonstrated that, on average, greater than 75% of the change in weight was due to accumulation of tumor mass. Pulmonary metastatic density was determined from each animal, and plotted as a function of change in weight (see figure 2). In contrast with previous reports [25], a moderate correlation was observed between approximate tumor mass and pulmonary metastatic density ($r = 0.48$).

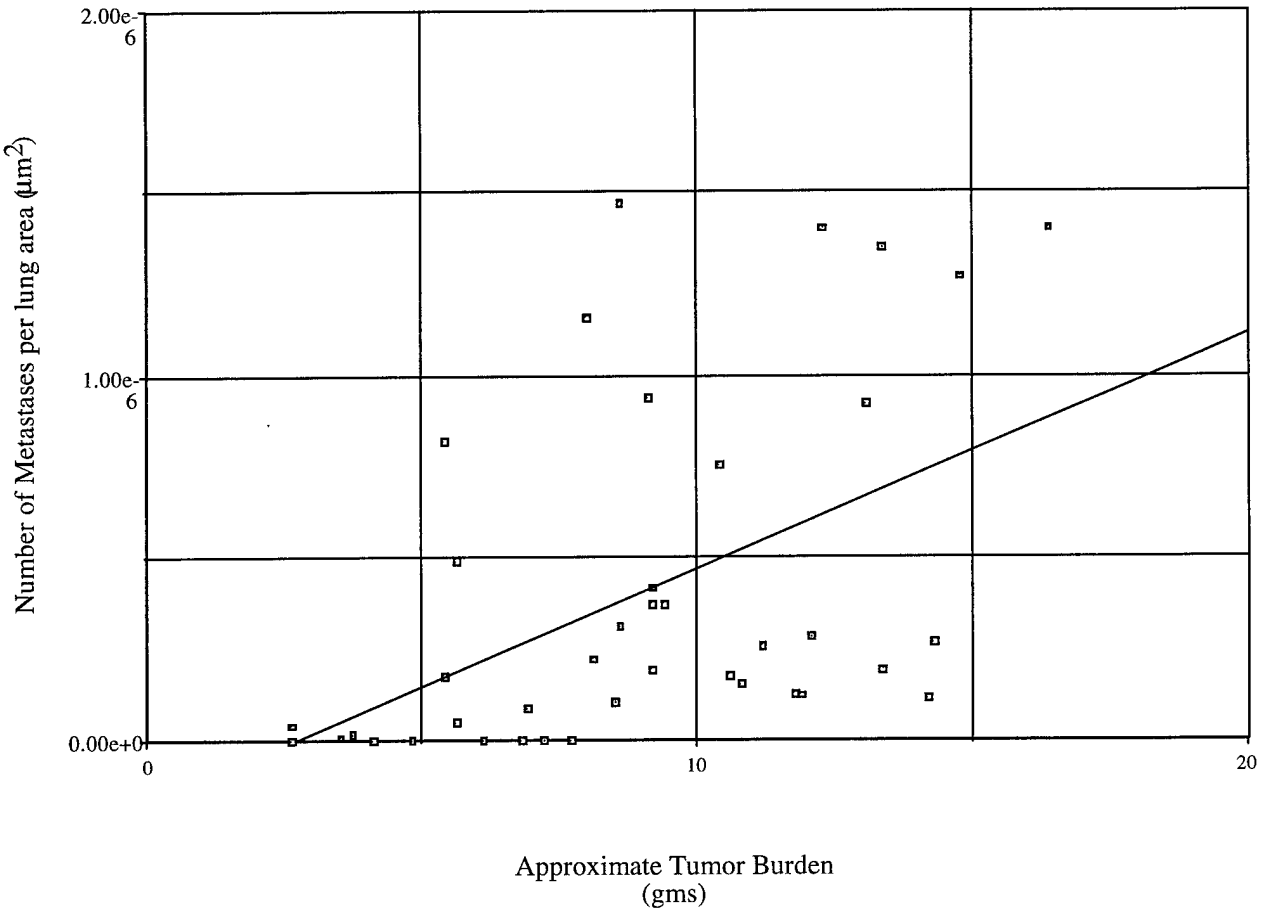

Figure 2: Correlation of Pulmonary Metastatic Density with Approximate Tumor Burden. The pulmonary metastatic density was measured as described and plotted versus the change in weight of the animal between diagnosis of the primary tumor and sacrifice.

Identification of Inbred Strains Bearing Metastasis Suppresser Gene(s):

To identify mouse inbred strains bearing dominant metastasis suppresser alleles, the FVB/N-TgN(MMTVPyMT) animal was bred to 27 different inbred strains to generate F_1 animals. The inbred partners were selected from the various branches of the phylogenetic tree of the laboratory mouse [26], to increase the likelihood of observing a strain specific variation in metastatic potential. In addition to the common inbred strains, representative strains from the more evolutionary diverged species *M. castaneous*, *M. molossinus*, and *M. musculus* have also been included. Virgin female F_1s were aged and monitored for the appearance of the primary tumor. Tumor bearing animals were maintained for 30-40 days after diagnosis, then sacrificed and autopsied. Representative animals from each mating were examined to determine whether metastatic organ tropism was altered. Primary tumor tissue was harvested to determine the transgene expression levels by northern blot analysis, and the lungs collected from each F_1 for histological examination as described previously. To control for the correlation between metastatic density and approximate tumor burden, the density of pulmonary metastases was normalized dividing the number of metastases per unit lung area by the approximate tumor burden, as defined by the change in weight.

To date, results have been obtained for 25 different inbred strain combinations. As expected, a range of results has been observed (see table 1). A number of strains demonstrated no significant variation from the FVB/N parent strain in the number or size of the pulmonary metastases. However, 14 different inbred strains had a statistically significant reduction in pulmonary metastatic density compared to FVB/N. Six strains demonstrate a 2-3 fold reduction, and the remaining 4 strains exhibit a 4-16 fold reduction. At present, none of the strains analyzed have demonstrated a significant increase in pulmonary metastases, suggesting the lack of a dominant metastatic enhancer allele in the inbred strains tested, relative to FVB/N. Preliminary northern blot analysis of the PyMT transgene in the primary tumor indicate that there is not significant variation in expression between the FVB/N parent and the F_1s, suggesting that the reduction in pulmonary metastatic density is not due to alterations in expression of the transgene. Ten strains accumulated statistically significantly less tumor burden, as measured by the average change of weight between diagnosis and sacrifice (see table 2). The decrease in tumor tissue accumulation in these strains was the result of a combination of fewer tumors per animal as well as a decrease in tumor size. The change in the metastatic index of these strains might therefore be due to genetic effects on tumor initiation or outgrowth rather than directly on metastatic potential. Sixteen strains accumulated tumor mass at an equal or greater amount than the FVB/N parental strain. The decrease in metastatic index in these strains is therefore likely to be due to a direct effect on metastatic progression, rather than an earlier stage of tumorigenesis.

Preliminary Conclusions:

These preliminary results strongly support the applicability and feasibility of the mouse as a model system for the identification of mammary cancer metastasis suppresser genes. The availability of high resolution mouse genetics and the ability to generate large numbers of backcross or intercross animals make the mouse uniquely useful for the genetic dissection of complex quantitative traits like metastatic progression. Utilizing standard mouse genetic approaches, the genetic location of the metastatic suppressers can be relatively easily determined.

Statistically significant variations in pulmonary metastatic density were observed when the genetic background of the tumor was changed by generating outcross F_1 progeny (table 1). Alterations in transgene expression in the primary tumors were not observed by northern blot analysis when comparing the FVB/N and F_1 transgenic animals, suggesting that the variation in pulmonary metastatic density was due to the presence of a metastasis suppresser or modifier gene in the non-FVB/N parent. Significant suppression of pulmonary metastatic density was observed in the F_1 progeny of three inbred strains, DBA/2J, KK/HiH, and NZB/B1NJ. No differences were observed in the number of primary tumors or the average tumor burden. Therefore, the decrease in the metastatic index in these strains is likely to be due to the presence of a metastatic suppressor gene(s) rather than a secondary effect of altered tumor initiation or growth (table 2). These three strains are distantly related, DBA/2J and NZB/B1NJ separated from common laboratory progenitors more than 50 years ago, and KK/HiJ derived from primarily Asian origin [27]. As a result of the distant relationship between these three strains, it is not possible to postulate whether the same metastatic suppresser gene(s) is present in all of the strains, or whether a different gene(s) is responsible for the phenotype observed. The range of results

observed would suggest that the metastatic suppression observed is likely due to multigenic interactions. Genetic mapping of the gene(s) responsible for the metastatic suppression in each of the strains will be required to elucidate the number genes involved.

Work In Progress

Since the F_1 hybrid animals produced from inbred mouse strains DBA/2J and NZB/B1NJ demonstrated significant reduction in the density of pulmonary metastases but accumulate identical amounts of tumor tissue, with indistinguishable kinetics as the FVB/N animals, these strains have been selected for quantitative trait mapping experiments. Two different strategies are currently being pursued to map the genetic location of the metastatic suppressor loci. Preliminary low resolution mapping of the metastatic suppressor gene (or genes) in the DBA/2J strain is being carried out using the AKXD recombinant inbred mapping panel. Recombinant Inbred (RI) strains are mice that are derived from an outcross between two progenitor strains, for example AKR/J and DBA/2J, followed by an intercross. The F_2 animals are then randomly chosen and separated, then more than 20 generations of brother-sister matings performed. The result is a series of inbred strains whose genomes are mosaics of the progenitor strains, each line having a unique set of genes from each of the progenitor strains. Every RI strain therefore has a unique set of recombination sites distributed randomly throughout its genome. A set of RI strains can therefore be used essentially in the same manner as a mapping cross to determine linkage. RI strains have been very valuable for the analysis of complex traits, since a major advantage of RI analysis of complex traits is the ability to replicate the phenotype in unlimited number of animals for each RI genotype [28]. This permits the minimization of errors due to environmental or experimental variation by combining data from large numbers of animals to obtain a mean value for each RI genotype. This disadvantage of this strategy is that the small number of available RI genotypes do not provide sufficient statistical rigor to definitively map complex traits that are controlled by more than three or four loci. Nonetheless, RI analysis can readily provide suggestive identification of genomic regions that contribute most strongly to the phenotype [29-31], which can subsequently be confirmed by backcross or intercross analysis. RI analysis is particularly useful because the segregation of the two parental genomes in each of the common RI strains has already been determined. It is therefore possible to generate approximate genetic maps by determining the segregation of the phenotype in each RI line and comparing it to the pre-existing strain distribution pattern, without having to perform additional genotyping.

The second strategy currently in progress is the development of intraspecific backcrosses between FVB/N and both NZB/B1NJ and DBA/2J. Since an appropriate recombinant inbred panel does not exist for NZB/B1NJ, the generation of a backcross panel is required for the genetic mapping of the loci responsible for the suppression of the metastatic phenotype. In addition, due to the limited resolution of the recombinant inbred panel analysis, a backcross panel is being generated between FVB/N and DBA/2J to confirm the results of the recombinant inbred mapping experiments and to refine the genetic localization.

Table 1: Affect of Maternal Genotype on Pulmonary Metastatic Density

Maternal Genotype	Metastatic Index	Standard Deviation	N	Metastatic Index Relative to FVB/N	p value versus FVB/N
FVB/N	3.31E-07	4.44E-07	60	1.00	1.00
RF/J	8.63E-09	1.93E-08	5	0.03	ND
C58/J	2.17E-08	4.08E-08	19	0.07	1.63E-04
C57BR/cdJ	2.35E-08	4.16E-08	16	0.07	1.52E-03
NZB/B1NJ	2.68E-08	3.82E-08	23	0.08	1.82E-04
I/LnJ	8.78E-08	1.75E-07	32	0.27	2.37E-04
DBA/2J	4.30E-08	6.64E-08	14	0.13	5.02E-04
KK/HiJ	6.09E-08	6.71E-08	13	0.18	1.19E-03
MOLF/Ei	5.21E-08	6.99E-08	9	0.16	1.10E-03
SEA/GnJ	8.39E-08	1.12E-07	23	0.25	2.82E-03
NZW/LacJ	1.24E-07	2.01E-07	10	0.37	0.02
CE/J	1.27E-07	1.42E-07	11	0.38	0.06
ST/J	1.37E-07	2.01E-07	11	0.41	3.47E-03
C57BL/6JNIcr	1.25E-07	3.02E-07	41	0.44	0.01
P/J	1.58E-07	1.14E-07	15	0.48	0.10
DBA/1J	1.66E-07	4.09E-07	10	0.50	0.21
NOD/LtJ	1.68E-07	1.40E-07	19	0.51	0.02
A/J	2.00E-07	2.16E-07	6	0.61	0.18
C3H/HeNIcr	2.02E-07	2.48E-07	20	0.61	0.08
SWR/J	2.46E-07	3.93E-07	21	0.74	0.30
CBA/CaJ	2.94E-07	2.36E-07	12	0.89	0.51
BUB/BnJ	4.35E-07	3.39E-07	14	1.32	0.50
129/J	4.47E-07	3.21E-07	12	1.35	0.13
BALB/cAnNIcr	4.51E-07	2.72E-07	18	1.36	0.33
C57BL/10J	6.71E-07	8.58E-07	13	2.03	0.23
CAST/Ei	6.75E-07	9.61E-07	5	2.04	0.51
AKR/J	1.09E-06	1.12E-06	13	3.29	0.07
LP/J	0.00E+01	0.00E+01	4	0.00	ND

Metastatic Index = number of metastases/lung area (μm^2)/tumor burden
P value was determined by comparing the FVB/N results to the average metastatic index for each strain with Student's T-test.

Table 2: Comparison of Average Change of Weight in F_1 Outcross Animals

Maternal Genotype	Average Change in Weight (gms)	St Dev	Change Relative to FVB/N	P value vs FVB/N
LP/J	4.15	0.99	0.45	1.01E-05
CAST/Ei	5.64	1.79	0.61	5.44E-03
C57BR/cdJ	5.71	1.58	0.62	6.32E-05
RF/J	5.98	2.59	0.65	0.04
DBA/1J	6.32	2.52	0.68	7.33E-03
129/J	6.42	2.64	0.70	6.24E-03
SEA/GnJ	6.42	2.63	0.70	2.50E-03
MOLF/Ei	6.54	5.12	0.71	0.17
C57BL/6JNIcr	6.89	2.95	0.75	7.33E-02
C57BL/10J	7.62	2.63	0.83	0.41
C58/J	7.76	2.26	0.84	0.06
I/LnJ	7.78	2.65	0.84	0.05
KK/HiJ	8.25	6.04	0.89	0.56
AKR/J	8.43	2.78	0.91	0.90
A/J	8.43	3.06	0.91	0.58
SWR/J	8.65	3.92	0.94	0.55
BUB/BnJ	9.01	3.55	0.98	0.41
CE/J	9.07	2.76	0.98	0.88
FVB/N	9.23	2.09	1.00	1.00
NZB/B1NJ	9.31	2.46	1.01	0.91
P/J	9.47	2.42	1.03	0.78
DBA/2J	9.53	3.97	1.03	0.80
CBA/CaJ	10.18	3.18	1.10	0.39
ST/J	10.34	3.75	1.12	0.62
C3H/HeNIcr	11.20	4.08	1.21	0.06
NOD/LtJ	11.64	2.30	1.26	2.49E-03
BALB/cAnNIcr	11.65	4.78	1.26	0.09
NZW/LacJ	12.74	2.62	1.38	2.36E-03

P value was determined by comparing the FVB/N results to the average change of weight for each strain with Student's T-test.

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Personnel

Kent W. Hunter, Ph.D.	Principal Investigator